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A STUDY OF THE GROWTH IN CULTURE OF VERTICILLIUM ALBO-ATRUM, B. ET BR.

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A Study of the Growth in Culture of Verticillium albo-atrum, B. et Br.

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With twelve Figures in the Text.

THIS paper deals with the growth of *Verticillium albo-atrum*, B. et Br., with special reference to the effect of temperature and aeration. A comparison is also made of the method of estimating growth by surface spread of the fungus on a solid medium with a method of dry-weight determination.

The later portion of the paper deals with zone formation, and an attempt has been made to determine some of the conditions which bring about such formation.

The original culture of *V. albo-atrum* was received from Dr. Bewley of Lea Valley Experiment Station, where it is a cause of wilt in tomatoes.

In most of the experiments described, the fungus has been grown in Coon's (1) medium; so the effect of this medium in various dilutions and of its chief constituents on the growth of the fungus was first studied. The fungus also grows well in oat broth, oatmeal agar, wheat broth, prune juice agar, tomato-extract agar, potato-extract agar, and potato-mush agar. It grew in Dox's or Czapek's solution, and also in Richard's synthetic solution. With solid media, agar was added in a concentration of $1\frac{1}{2}$–2 per cent., according to the acidity of the solution; with prune juice 3 per cent. agar was found necessary to ensure setting of the medium.

Very luxuriant growth takes place in potato mush and potato extract, and also on cooked banana and oatmeal agar. In Dox's solution and in corn-meal agar, it grew rather indifferently.

The sizes of the conidia varied with different media and with the liquid or solid nature of the medium. Sometimes the conidia, on germination in the hanging drops of water, produced abundant small secondary conidia; the average size of these was $4{\cdot}5\,\mu \times 2{\cdot}5\,\mu$, while that of ordinary conidia was $6{\cdot}8\,\mu \times 2{\cdot}5$–$5\,\mu$.

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Coon's medium (1) consists of $MgSO_4$, KH_2PO_4, asparagin, and maltose. Coon advises the steaming of the solution on three successive days, but no difference in the nutritive value of the medium was to be observed between steaming and sterilizing at 120° C.

V. albo-atrum grew very well in Coon's normal solution. No significant difference in the dry weight of the growth occurred when, in a series of experiments, the asparagin was reduced by half; but whenever the maltose is reduced, the reduction in growth is very great. When maltose is present in full strength the reduction of asparagin does not make much difference; again, if maltose is decreased to half neither can the addition of asparagin in full strength enhance the growth nor the reduction of asparagin diminish it.

The following gives the actual dry weight of the growth of *Verticillium* in Coon's medium with varying amounts of maltose and asparagin. These figures have been arrived at by estimating the dry weight of the fungus after sixteen days' growth on various modifications of Coon's medium with agar. The fungus was incubated at room temperature (18°–20° C.). The method of determining the dry weight is given later.

Normal Coon.	*Coon's with full maltose and half asparagin.*	*Coon's with half maltose and full asparagin.*
0·0460 grm.	0·0380 grm.	0·0303 grm.
0·0363 ,,	0·0313 ,,	0·0241 ,,
0·0324 ,,	0·0267 ,,	0·0221 ,,
0·0262 ,,	0·0182 ,,	0·0185 ,,
0·0252 ,,	0·0173 ,,	0·0170 ,,
0·0242 ,,	0·0164 ,,	0·0158 ,,
Av. 0·0317 ± 0·002 grm.	Av. 0·0246 ± 0·0019 grm.	Av. 0·0213 ± 0·0012 grm.

Coon's with half maltose and half asparagin.	*Coon's with full maltose and no asparagin.*	*Coon's with no maltose and full asparagin.*
0·0302 grm.	0·0271 grm.	0·0166 grm.
0·0231 ,,	0·0239 ,,	0·0102 ,,
0·0205 ,,	0·0234 ,,	0·0084 ,,
0·0183 ,,	0·0230 ,,	0·0076 ,,
0·0167 ,,	0·0225 ,,	0·0074 ,,
0·0155 ,,	0·0203 ,,	0·0066 ,,
Av. 0·0207 ± 0·0013 grm.	Av. 0·0234 ± 0·0006 grm.	Av. 0·0094 ± 0·0008 grm.

Coon's with half maltose and no asparagin.	*Coon's with no maltose and half asparagin.*	*Coon's with neither maltose nor asparagin.*
0·0297 grm.	0·0124 grm.	0·0074 grm.
0·0277 ,,	0·0084 ,,	0·0040 ,,
0·0220 ,,	0·0074 ,,	0·0040 ,,
0·0149 ,,	0·0067 ,,	0·0038 ,,
0·0148 ,,	0·0066 ,,	0·0036 ,,
0·0132 ,,	0·0061 ,,	0·0036 ,,
Av. 0·0204 ± 0·0018 grm.	Av. 0·0079 ± 0·0008 grm.	Av. 0·0044 ± 0·0004 grm.

The above data are represented graphically in Fig. 1, and here the importance of maltose is very manifest.

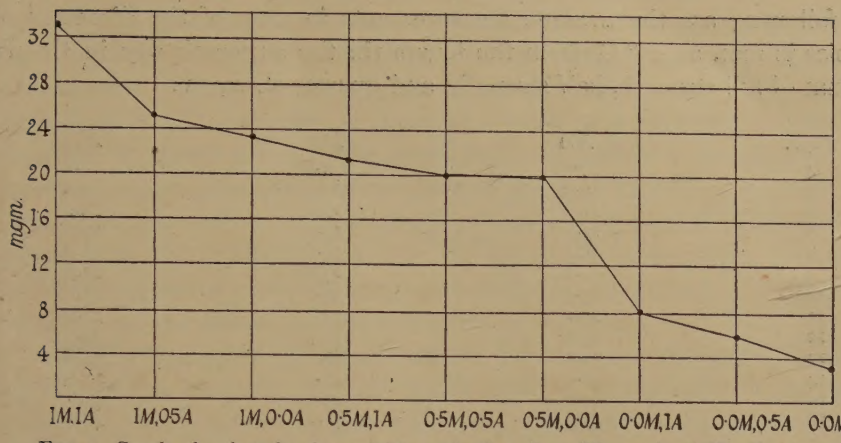

FIG. 1. Graph, showing the dry-weight production (in milligrams) of *V. albo-atrum* grown for sixteen days in Coon's medium with varying amounts of maltose and asparagin. 1 M = normal amount of maltose, 1 A = normal amount of asparagin, 0·5 M = half the normal amount of maltose, &c.

THE EFFECT OF AERATION AND OF TEMPERATURE ON THE GROWTH OF THE FUNGUS.

Method. The various temperature conditions were obtained by means of incubators for the higher temperatures, viz. between 14° and 30° C. For the lower temperatures, 5°–10° C., an automatically regulated refrigerator was used, and for 12° C. a cool storage room. Experiments on aeration were first carried on with flasks of different capacity containing the same quantity of the liquid medium. It was found more growth took place in the flasks with greater capacity. Next, flasks of same size were taken, and were aerated once every day for a few minutes. This gave no better growth than no aeration. Successful results were obtained by aerating very gently but continuously. This was done by means of a glass blower attached to the water tap. About fifty flasks were aerated equally and simultaneously. The main tube was connected with a flask having five branch tubes; these being connected to small jars with ten branch tubes, which were connected with the flasks to be aerated.

Throughout the course of experiment, data have been obtained both by measurement of the superficial growth on agar plates, and by determination of dry weight; and an attempt has been made to determine how far the spread of a fungus on an agar plate is a satisfactory measure of its rate of growth.

By measuring at regular intervals the spread of the fungus on an agar medium it has been found that *there is little or no difference in the rate of spread between Coon's normal, half-normal, and quarter-normal medium, though there is a marked difference in the amount of mycelium produced* (p. 534).

The following are the average measurements in mm. of the diameter of colonies in mm. at 25° C. from the fourth till the sixteenth day in Coon's medium of full strength, half strength, and quarter strength:

Days.	*N*/1	*N*/2	*N*/4
4	7·5	7·8	8·3
5	9·5	10·0	10·3
6	12·5	13·2	13·2
7	15·0	17·0	17·0
8	18·2	19·8	19·8
9	21·8	23·8	23·8
10	25·5	27·0	27·0
11	29·2	31·0	31·0
12	33·0	34·5	34·5
13	36·8	38·0	38·6
14	40·0	41·8	41·6
15	43·8	45·0	44·6
16	47·5	48·2	48·6

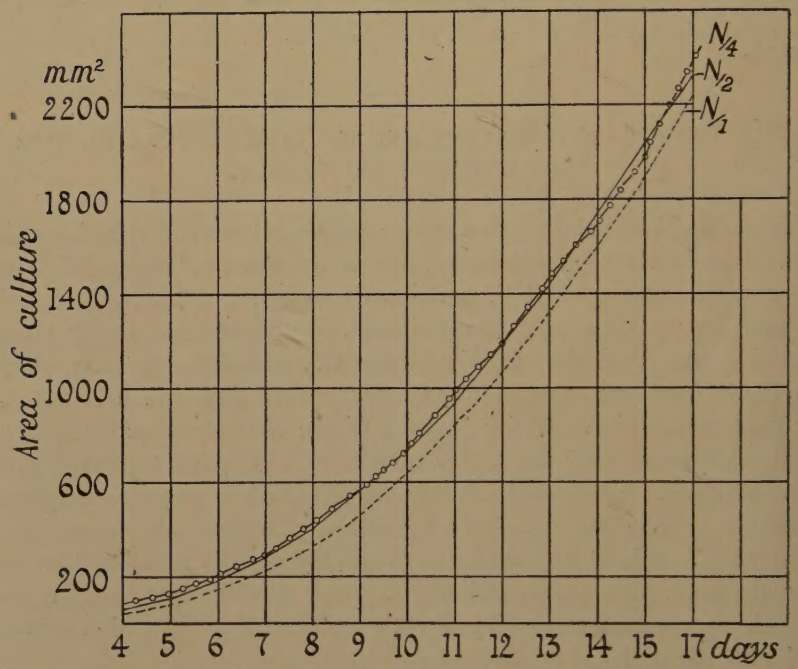

FIG. 2. Growth of *V. albo-atrum* on the different strengths of Coon's medium with agar. The broken line represents growth on full strength (N/1), the continuous line that on half strength (N/2), and the dotted line that on quarter strength (N/4). The growth is measured by the area of the plate covered by the mycelium; the area is estimated in terms of the square of the diameter measured in mm.

Fig. 2 shows the result graphically. It depicts surface growth of the fungus as determined by taking the squares of the diameter of the circular area covered by the fungus growing at 25° C. The rate of growth in area is seen to increase with the increasing diameter of the mycelium, thus following approximately the compound interest law.

Dry-weight determination. The procedure employed in determining dry weight is as follows: Weighing bottles with a filter-paper inside were placed in a drying oven at 65° C. for twenty-four hours, then cooled in a desiccator and weighed carefully to a tenth of a milligram. The liquid medium is passed through the filter-papers placed in a funnel, care being taken to scrape out any mycelium that might stick to the sides of the flask. The filtrate is then washed in distilled water, and placed in the weighing bottle and put in the drying oven at 65° C. for three days. The bottles are then placed in a desiccator and the weights determined. The difference is taken as the actual weight of mycelium produced.

In the case of an agar medium, the portion over which the fungus has spread out is carefully cut out and placed in a test-tube, with distilled water, which is boiled to melt the agar; the whole is then passed through the filter. Washing in this case is done with boiling distilled water three or four times. After that the material is dried and weighed as before.

The washing, especially with hot water, no doubt dissolves away a certain portion of the dry matter, but the amount so lost is slight, and is probably roughly proportional to the original mass of material.

Growth and Temperature.

Effect of temperature. The fungus grows very well both in darkness and light at room temperature in summer (18°–20° C.), whereas in winter little or no growth takes place at room temperature. Series of cultures in various media were grown at different temperatures, viz. 5°, 10°, 12°, 14°, 16°, 18°, 20°, 21°, 22·5°, 24°, 25°, 27°, and 33° C. It is found that the best growth takes place at 22·5° C. No growth took place in cultures incubated at 33° C. for a week, and when removed to 22·5° C. such cultures showed no growth; apparently the fungus had been killed or lost the power of germination. No growth took place in a week at 5° C., but when transferred to 22·5° C. the growth of such cultures was very vigorous. Even at 10° C. no apparent growth took place. Above that, growth begins, reaching its optimum at 22·5° C. At 25° C. the growth is less, and at 27° C. there is no growth.

Fig. 3 represents graphically the growth at different temperatures, determined first by measurement of the diameters of colonies after fifteen days, secondly by measuring the length of the germ tubes in hanging drops of distilled water after twenty-four hours. In measuring the length of the germ tube, all the tubes from a single spore were counted.

The two curves are very similar and have their optimum at 22·5° C. The difference lies in their maximum, for many spores produced short germ tubes at 27° C. in hanging drops, whereas when examining the agar medium of Coon's normal solution, no visible growth was found after fifteen days, though the spores remain living, for at a lower temperature a mycelium soon appears.

Measurement of spread of colonies at different temperatures after fifteen days:

	10° C.	12° C.	14° C.	16° C.	18° C.	21° C.	22·5° C.	25° C.	27° C.
Average diameter in mm.	0	10	14	35	45	55	60	45	0

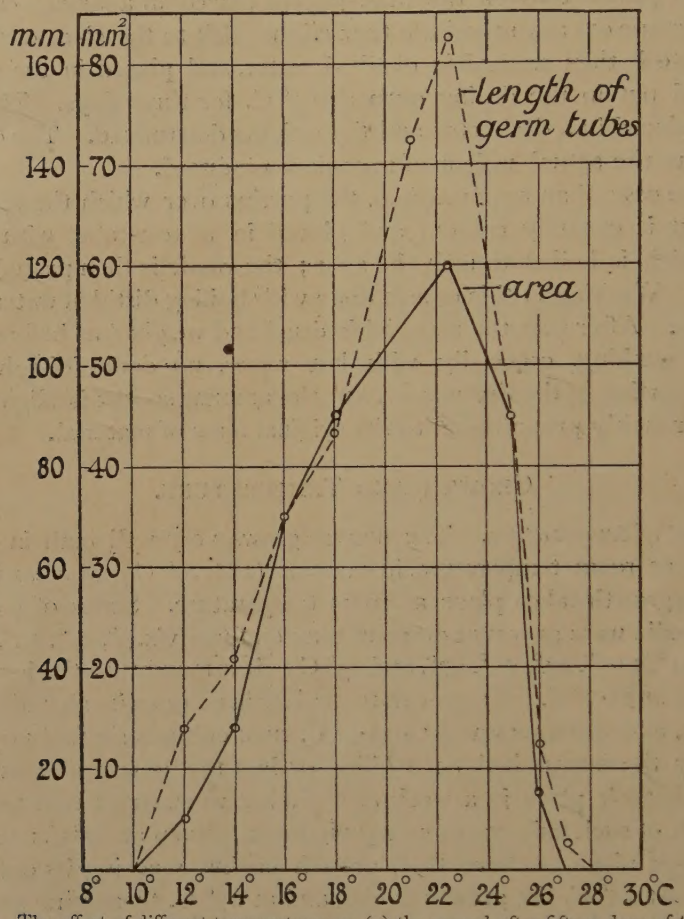

FIG. 3. The effect of different temperatures on (1) the spread after fifteen days of the mycelium on Coon's medium in terms of the diameter of the circular area covered, (2) the length of germ tubes developing from conidia after twenty-four hours in hanging drops. The right-hand vertical scale is that of the length of the germ tubes, the left-hand that of the diameter of the mycelial areas.

Measurement of germ tubes after twenty-four hours' growth, the unit being 0·5 μ:

12° C.—16, 16, 15, 18, 12, 12, 14, 10, 7, 8, 10, 15, 10, 8, 10, 16, 12, 8, 10, 15, 25, 25, 15, 20, 28, 18, 15, 18, 18, 15, 12, 15, 10, 10, 10, 8, 15, 18, 20, 12. Average, 14 = 28 μ.

14° C.—30, 32, 24, 15, 15, 22, 28, 25, 30, 18, 16, 14, 12, 20, 15, 25, 18, 30, 25, 10, 10, 12, 20, 22, 22, 18, 18, 20, 20, 18, 22, 30, 30, 22, 30, 12, 18, 12, 15, 30. Average, 21 = 42 μ.

16° C.—42, 45, 48, 25, 35, 28, 28, 55, 25, 40, 45, 50, 35, 40, 45, 55, 38, 22, 32, 40, 20, 25, 28, 24, 30, 35, 20, 60, 50, 55, 25, 20, 40, 35, 30, 25, 45, 50, 40, 35. Average, 36 = 72 μ.

18° C.—40, 25, 20, 25, 45, 40, 35, 30, 30, 45, 45, 35, 40, 45, 50, 55, 60, 60, 45, 40, 50, 60, 35, 40, 28, 35, 40, 45, 65, 65, 28, 45, 50, 35, 40, 38, 45, 55, 40, 55. Average, 43 = 86 μ.

20° C.—65, 60, 80, 65, 65, 70, 55, 85, 80, 75, 80, 60, 75, 80, 85, 45, 50, 90, 90, 85, 65, 75, 70, 85, 60, 60, 70, 80, 85, 95, 90, 75, 60, 65, 80, 75, 55, 40, 45, 62, 38. Average, 72 = 144 μ.

22·5°C.—64, 80, 95, 110, 120, 65, 70, 80, 90, 80, 85, 60, 80, 80, 60, 50, 125, 100, 75, 120, 80, 75, 80, 100, 65, 85, 90, 100, 55, 60, 80, 70, 75, 65, 95, 90, 130, 68, 70. Average, 82 = 164 μ.

25° C.—30, 35, 40, 45, 45, 30, 35, 32, 45, 45, 50, 40, 35, 40, 45, 30, 35, 40, 45, 60, 65, 45, 50, 35, 50, 50, 45, 45, 30, 35, 45, 50, 40, 45, 40, 50, 75, 60, 70, 45, 50. Average, 45 = 90 μ.

27° C.—6, 30, 18, 13, 6, 10, 8, 8, 6, 6, 10, 8, 13, 16, 18, 6, 6, 8, 10, 8, 8, 10, 6, 13, 18, 10, 13, 8, 16, 6, 6, 6, 6, 8, 8, 11, 16, 18, 10, 8. Average, 10 = 20 μ.

Edson and Shapovalov (2), in their experiments to determine the growth of various fungi at various temperatures, found for *V. albo-atrum* an optimum at 25° C. They incubated the fungus at 10°, 15°, 20°, 25°, and 30° C. If, however, they had experimented with smaller differences of temperature, as has been done in this paper, the optimum might have been found to be between 20° and 25° C. or between 25° and 30° C.; and the growth at the real optimum might have been considerably greater than that found at 25° C., which was arbitrarily assumed to be the optimum.

FIG. 4. Photograph showing the difference in the amount of mycelial 'spread' due to difference in thickness of the medium, that on the left hand being the thicker. It also shows the difference in zonation produced by the difference in thickness. Incubated at 25° C.

It may be mentioned that with the fungus here investigated the *maximum* varied with different food material, as will be described later on, but the *optimum* always remained the same.

Edson and Shapovalov determined the optimum by measuring the spread of the fungus on solid media. It should be pointed out, however, that with this method great care must be taken that the thickness of the medium is constant; for the spread of the fungus varies with thickness unless the medium is very thick, i.e. about 10 mm., in which case a little difference does not affect the growth. Fig. 4 shows a photograph of halves of two plates containing media, 4 and 6 mm. thick respectively. The difference in size of the fungal growth is well marked.

It was found that *V. albo-atrum* could under no circumstances withstand 33° C. for one week. An attempt was therefore made to discover how long it could withstand high temperatures. The period was gradually decreased and various food materials used. It was found that the lethal temperature varied with different media. Thus with potato-mush agar, pea-broth agar, Dox agar, corn-meal agar, and cooked banana exposed to 33° C.

for forty-eight hours, there was no survival, but on pea agar, corn-meal agar, and prune agar it survived the temperature for twenty-four hours, but not on potato mush or tomato mush. When, however, the period was reduced

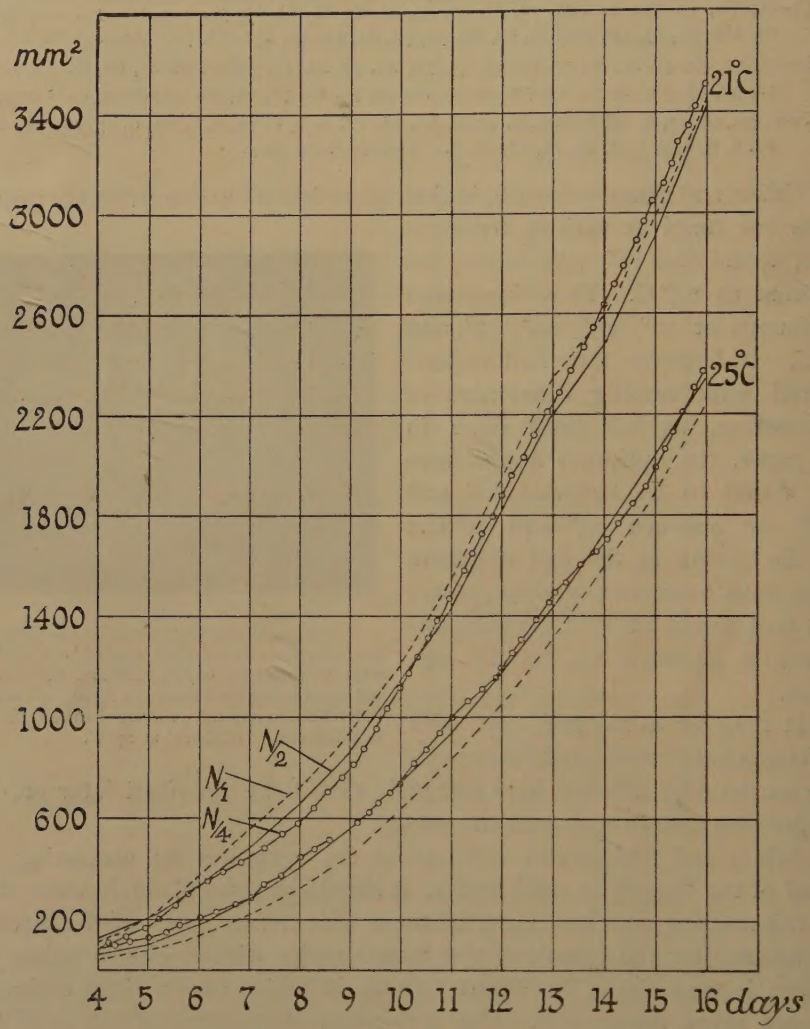

FIG 5. Curves showing the relative area (diameter in mm. squared) covered by the mycelium at 21° C. and 25° C. in agar cultures of Coon's medium, full strength (N/1), half strength (N/2), and quarter strength (N/4).

to eighteen hours it survived on all the media. The power of withstanding high temperatures agrees to a certain extent with that recorded for the two strains used by Edson and Shapovalov, though both had a higher maximum temperature. They write: 'The two strains do not survive exposure for several days to a temperature of 35° C. or higher;' and again: 'With

sufficient moisture present no growth took place in 30° or 35° C. after seven, fourteen, or twenty-one days.'

The usual bacterial method was followed to determine the thermal death-point of the fungus. A number of tubes of melted potato-mush agar were inoculated and kept at 40° C. for ten minutes and then poured into sterile Petri dishes and cooled rapidly. Next the temperature of the bath was raised to 43° C., and another set of tubes inoculated and kept there for ten minutes, and then poured into sterile Petri dishes and cooled rapidly. Similar experiments were made at 46°, 49°, 52°, and 55° C. These Petri dishes were incubated at 22·5° C., the optimum temperature for this fungus. Innumerable colonies appeared on the second and third day from those exposed to 40°, 43°, and 46° C. A smaller number of colonies appeared at 49° C. No colonies appeared at 52° C. Next a similar set of experiments were made at 48°, 50°, 52°, and 54° C. A number of colonies appeared at 48° and some at 50° C. One or two colonies appeared in some cases at 52° and none at all at 54° C. So the thermal death-point may be put between 52° and 54° C. for a ten minutes exposure.

Growth in Media of Different Concentration at One Temperature.

It has been found that if certain amounts of food material are present the increase in diameter of the mycelium is fairly constant for a particular temperature, provided that the thickness of the medium is the same in all cultures. Thus a colony will spread equally in Coon's normal, half normal, and quarter normal, if incubated at the same temperature. The following are the actual measurements (squares of the diameter in mm.) for Coon's N/1, N/2, and N/4 at temperatures 21° and 25° C. It will be seen from Fig. 5 that spread is equal for different concentrations at the same temperature.

21° C.				25° C.			
Days.	*N*/1	*N*/2	*N*/4	*Days.*	*N*/1	*N*/2	*N*/4
4	81	100	77	4	56	60	68
5	182	188	150	5	90	100	106
6	361	324	324	6	150	175	182
7	529	494	461	7	225	289	270
8	702	665	575	8	330	319	410
9	930	900	870	9	475	560	560
10	1225	1135	1135	10	650	729	729
11	1560	1444	1482	11	860	961	990
12	1936	1831	1849	12	1089	1190	1190
13	2304	2190	2227	13	1354	1444	1480
14	2601	2570	2650	14	1600	1740	1720
15	3025	3100	3080	15	1916	2025	1980
16	3481	3457	3600	16	2240	2325	2350

In one case, however, the effect of a difference of temperature was not manifest. This was with a six-months-old solution (Coon's normal) in which growth had previously stopped; the pH was 8·2. This medium was filtered,

agar added, and then plated. A very thin mycelium was produced, only just visible to the naked eye, but this grew very rapidly, and there was no difference in the diameters of the colonies incubated at the two temperatures. The following are the measurements in mm. from the fourth till the seventeenth day:

	21° C.				25° C.			
Days.	*Pl. I.*	*Pl. II.*	*Pl. III.*	*Pl. IV.*	*Pl. I.*	*Pl. II.*	*Pl. III.*	*Pl. IV.*
4	10·0	9·0	9·0	8·0	10·0	9·0	10·0	10·0
5	14·0	13·0	12·0	12·0	14·5	12·5	13·0	14·0
6	18·0	16·0	17·0	17·0	16·5	16·0	16·0	17·0
7	22·0	21·0	21·0	21·0	22·0	21·0	20·0	21·0
8	26·0	26·0	26·0	25·0	26·0	26·0	24·0	25·0
9	30·5	29·0	29·0	28·0	31·0	29·0	27·0	30·0
10	34·0	33·0	33·0	33·0	36·0	32·0	32·0	34·0
11	38·5	37·0	37·5	37·5	39·0	37·0	37·0	38·0
12	41·5	40·0	40·0	40·5	42·5	40·0	40·0	41·5
13	45·5	45·0	45·0	45·5	46·0	45·0	43·0	45·0
14	50·0	51·0	50·0	49·5	49·5	48·5	47·0	49·0
15	54·5	54·0	54·5	53·5	53·0	62·0	52·0	53·0
16	58·5	57·0	57·5	57·0	57·0	56·0	56·0	56·0
17	61·5	62·0	61·5	60·0	60·0	60·0	59·5	60·0

Relation between Rate of Surface Spread of the Fungus and its Increase in Weight.

The amount of spread may be taken as a satisfactory measure of growth when determining the effect of temperature with a definite medium of constant thickness. But when growth-data at a given temperature for different media are required it gives fallacious results. Almost the same rate of spread is found with normal N/2, N/4, N/8, and N/16 Coon's medium, though the determinations of dry weight show the increase in fungal material to be very different. The difference is especially marked for growth in liquid media.

Fig. 6 shows graphically the relation between area and dry weight in Coon's medium with agar at a temperature of 22° C. The diameters were measured on the 1st, 4th, 8th, 11th, 14th, and 17th days, when also eight plates at a time were taken for dry-weight determination. The following gives an average figure for the area (diameter in mm. squared) and dry weight:

1st Day.
Area . . . . . 9 mm.²
Weight . . . . . Nil.

4th Day.
Area . . . . . 100 mm.²
Weight . . . . . 0·0015 ± 0·0003 grm.

8th Day.
Area . . . . . 900 mm.²
Weight . . . . . 0·0148 ± 0·0012 grm.

11th Day.
Area . . . . . 1,764 mm.²
Weight . . . . . 0·0254 ± 0·0014 grm.

14th Day.
Area . . . . . 3,025 mm.²
Weight . . . . . 0·0380 ± 0·0021 grm.

17th Day.
Area . . . . . 4,900 mm.²
Weight . . . . . 0·0557 ± 0·0032 grm.

It will be seen from the graph that the area curve more or less follows the compound interest law, at least for the period when the fungus is growing vigorously, but the dry-weight curve runs almost in a straight line.

The graph in Fig. 7 shows the increase in mycelial 'spread' obtained by

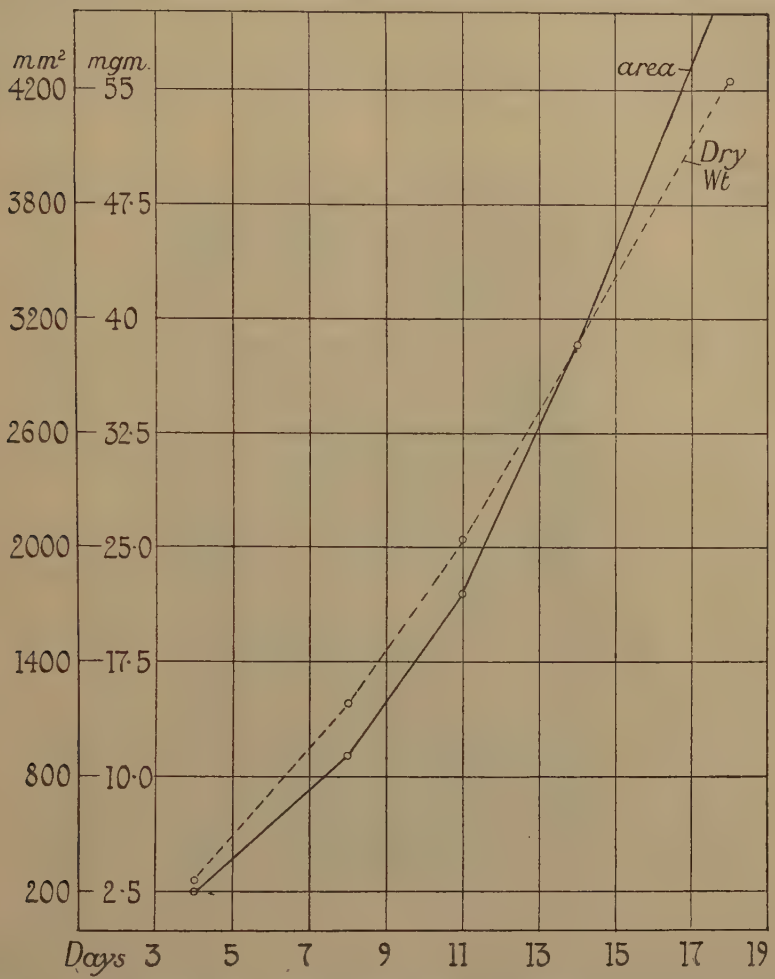

FIG. 6. Curves showing the relation between the daily increase in dry weight of *V. albo-atrum* in Coon's liquid medium, and the daily increase in 'spread' of the mycelium on the same medium with the addition of agar. The scale represents area covered by the mycelium as given in terms of the square of the diameter measured in mm. The vertical scale on the left represents mm.², that on the right milligrams.

measuring diameters daily up to ten days at different temperatures. At the end of that period the dry weight for the total growth was determined. The area curve and dry-weight curves are very much alike, and it is only in this case where we are estimating the effect of *different* temperatures that the spread is a satisfactory measure. By following the daily growth curves,

we find that the growth at the optimum temperature 22·5° C. is always the best, only it is not so marked during the first twenty-four hours, when almost equal amount of growth has taken place at 20° C. But when the spore germination curve is studied in Fig. 3 we find that the difference between 20° C. and 22·5° C. is quite significant even for the first twenty-four hours.

The following are the average figures for different temperatures of the

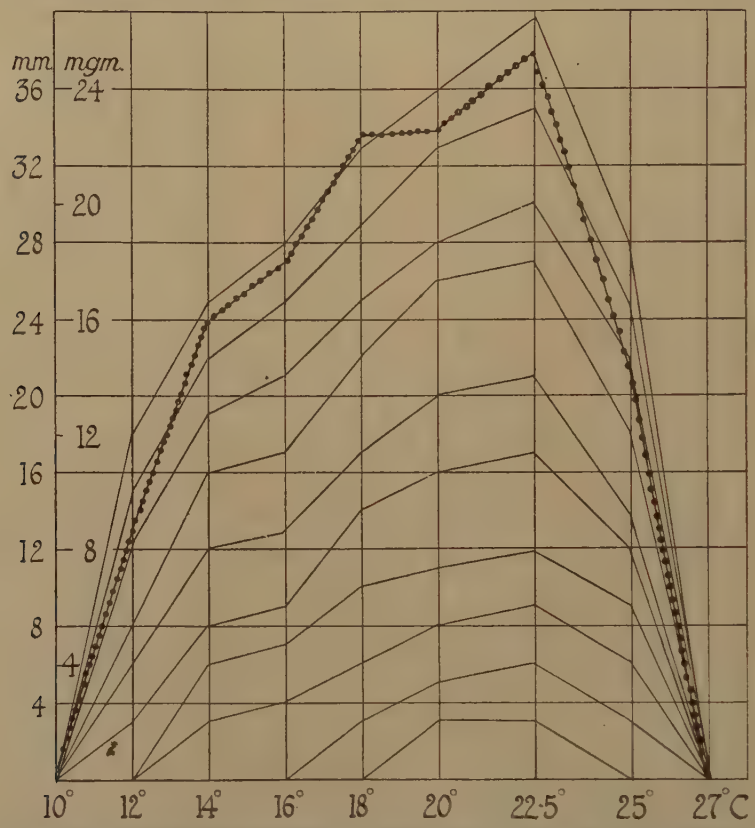

Fig. 7. The continuous lines represent the daily growth in *diameter* of mycelium on Coon's medium (plus agar) at the temperatures indicated. The thick dotted line represents the dry weight of mycelium produced in ten days at the different temperatures. The two vertical scales represent on the left mm., on the right mgm.

diameters of the mycelia taken daily. Fig. 8 shows a photograph of the colonies grown at different temperatures. It will be seen that no growth has taken place at 27° C. nor at 10° C.

The following data show the dry weight at different temperatures after a period of ten days; the average is that of three samples:

12° C.	14° C.	16° C.	18° C.	20° C.	22·5° C.	25° C.
0.0087 grm.	0.0161 grm.	0.0181 grm.	0.0224 grm.	0.0225 grm.	0.0253 grm.	0.0144 grm.

Aeration.

The effect of aeration was studied by aerating a series of culture flasks continuously, and determining at regular intervals the dry weight of mycelium produced. These experiments were all carried on at 21° C. in a large incubator, and the flasks were aerated with the help of a blast pump connected with the water supply. Control non-aerated flasks were placed in the same incubator. In the first set of experiments, the flasks were

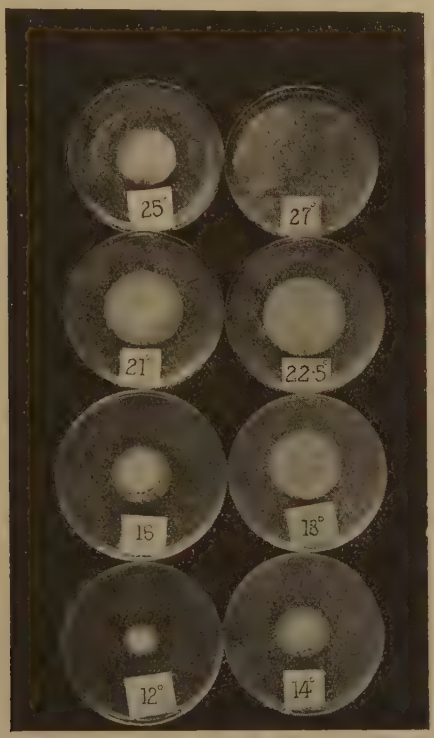

Fig. 8. Photographs of cultures of *V. albo-atrum* after fifteen days at 12°, 14°, 16°, 18°, 21°, 22·5°, 25°, and 27° C.

aerated discontinuously, once for three minutes daily, and at the same time a series of flasks were shaken. The aerated ones were found to show less growth than the non-aerated ones, and about the same as those shaken. For five successive weeks a number of flasks were taken, and dry weights determined, and each time the aerated ones gave less growth, as did the shaken flasks. Obviously, the fungal hyphae were torn by the direct shaking or by that caused by rapid bubbling and this retarded growth. It was also found in this connexion that the best growth had taken place by the

end of the second week, and that in the following two weeks there was very little growth.

A second set of experiments was then started in which air was gently bubbled continuously through the liquid in the flasks, and a set of eight flasks from the aerated ones and another set of eight from the non-aerated ones were taken away every five days for dry-weight determination for a period of fifteen days. The result was very striking; after the first five days the dry weight was nearly double that of the non-aerated ones. After ten days the difference was still very great; after fifteen days, how-

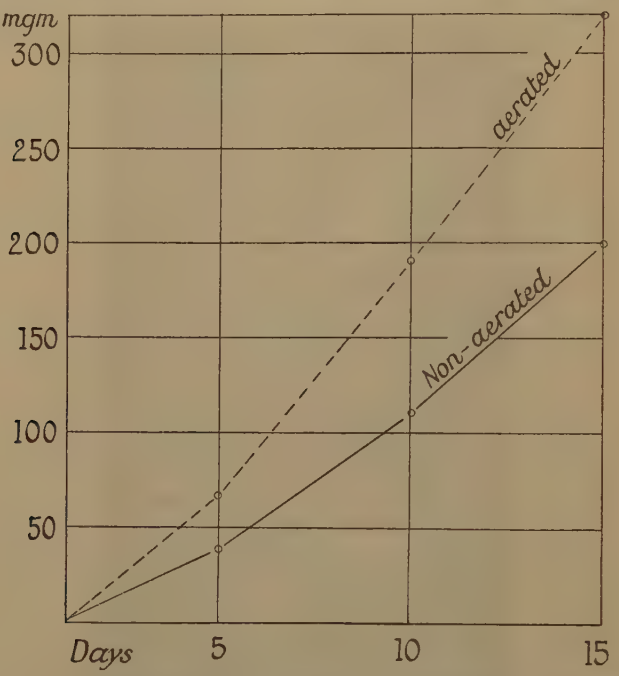

FIG. 9. Dry-weight production of *V. albo-atrum* in aerated and non-aerated cultures in Coon's medium.

ever, the ratio was falling. These results are represented graphically in Fig. 9. At the end of five days the aerated cultures weighed on an average $0{\cdot}0659 \pm 0{\cdot}0027$ grm., whereas the non-aerated ones gave only $0{\cdot}0381 \pm 0{\cdot}0028$ grm. After ten days the aerated gave $0{\cdot}1942 \pm 0{\cdot}006$ grm. and the non-aerated $0{\cdot}1107 \pm 0{\cdot}0056$ grm.; and after fifteen days $0{\cdot}3239 \pm 0{\cdot}0114$ grm., and $0{\cdot}2099 \pm 0{\cdot}0058$ grm. respectively. These figures, which are in all cases the mean of eight samples, show that the differences are very significant.

In this experiment all the media, viz. Coon's, pea broth, tomato juice, prune juice, potato extract, Richard's synthetic, &c., in which the fungus grew were rendered alkaline. The highest degree of alkalinity reached in Coon's medium was pH 8·2 after six months, the normal pH of the

medium being 4·3. No particular relation between growth and change of pH could be established. It was however observed that pH remains low when the media is aerated, though more growth takes place.

Experiments were next carried on to find out how long the weight continued to increase in the aerated and in the non-aerated cultures, and whether, when the growth had stopped in both, the total weight was the same. This experiment was carried on in two sections. In the first aerated and non-aerated flasks were inoculated and incubated at 21° C., and dry weight

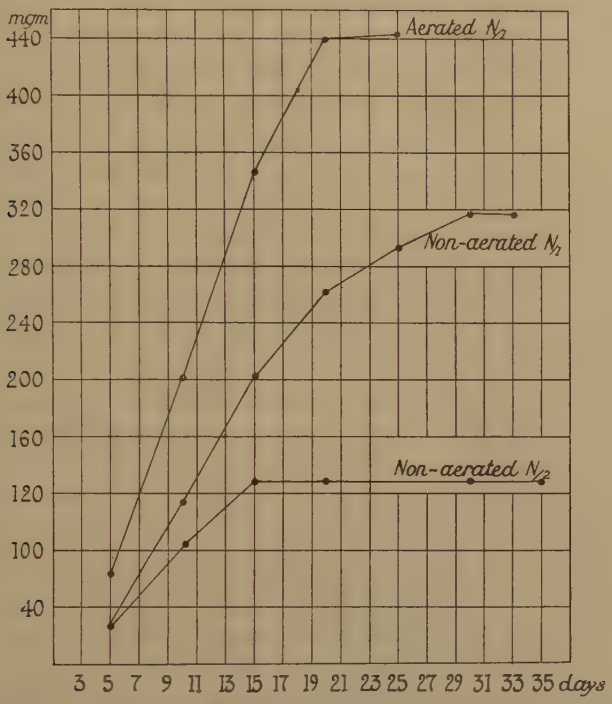

FIG. 10. Dry-weight production of *V. albo-atrum* in aerated and non-aerated Coon's medium of normal strength (N/1), and in non-aerated Coon's medium of half strength (N/2).

determined every five days for twenty-five days. Inoculations were made every time with equally old cultures, generally three weeks old ones on potato mush in tubes. Eight flasks were taken every five days for determination of the dry weight. In the third interval nine aerated flasks were lost accidentally, so for the remaining three intervals only five flasks could be examined at a time. This would cause a greater probable error, but fortunately the results were very good, the error being quite low. As the eighty flasks required for the two sets, aerated and non-aerated, could not be started at once owing to the limited space available, the experiment was begun first with aerated ones only (forty flasks), and as, at each interval of five days, eight were taken out, fresh non-aerated ones were put in their place,

so that when the last set of aerated ones were taken out and the last non-aerated ones put in, only another five days were required to complete the whole experiment. Every care was taken that the parent cultures were of the same generation and age.

We find by studying Fig. 10 that by the end of twenty days maximum growth had taken place in the aerated ones, for at the end of twenty-five days there was no increase of growth over that of twenty days.

In the non-aerated ones, however, maximum growth had not been reached by the end of twenty days, so they were carried on to thirty-five days. At the end of thirty days the dry weight still showed a certain gain, but by the end of the thirty-five days there was no further gain. So the maximum growth had taken place, and the total weight was much less than the total weight of the aerated ones. This shows the significant effect of aeration.

Another set of experiments was carried out at the same time to determine the actual difference in dry-weight production in a normal and half-normal medium; it had been found previously, on the bases of the rate of spread of the mycelium, that there was no difference. These flasks were not aerated, and were examined on the fifth, tenth, twenty-fifth, thirtieth and thirty-fifth days. We find very little difference in growth between the tenth and twenty-fifth days, and after that there is no further increase in growth. The total weight is only about a third of the normal. When this result is compared with the result obtained by the method of increase in diameter it is clear that the increase in surface area of a culture on a solid medium does not give a satisfactory measure of the growth of this fungus in media of different concentrations.

The following are the determinations of dry weight on Coon's normal liquid medium at 21° C. The figures are in all cases the means of a number of cultures; this number is given in the fourth column.

Coon's Medium (Full Strength).

Dry Weight (grm.).

Days.	*Aerated.*	*Non-aerated.*	*No. of samples.*
5	0·064 ± 0·003	0·037 ± 0·001	8
10	0·203 ± 0·004	0·115 ± 0·004	8
15	0·336 (5 samples)	0·205 ± 0·006	8
20	0·440 (5 samples)	0·262 ± 0·010	8
25	0·444	0·294	5
30	—	0·318 ± 0·003	8
35	—	0·318 ± 0·003	8

Coon's Medium (Half Strength).

5	—	0·037	5
10	—	0·085	5
25	—	0·129	5
30	—	0·130	5
35	—	0·128	5

In Fig. 10 the curves for the growth of the aerated and non-aerated mycelia during the early period when there was plenty of food material show that the growth does not follow the compound interest law, as does

the increase in surface area. Instead, there is approximately the same increment of weight for each period. This may be attributed either to the old mycelium ceasing to produce new growing hyphae or to the young mycelium, though increasing in amount, growing less and less vigorously.

An experiment was carried out to throw some light on the action of aeration. Does it act by oxidizing the staling products or by reducing their production? An experiment was started with a medium staled for thirty-five days which had been used for the non-aerated cultures and in which growth had stopped. The medium was filtered and put in flasks under aseptic conditions, and then inoculated and aerated for fifteen days. A similar set was left non-aerated. Very little growth took place in either, and the growth in the aerated ($0{\cdot}0034 \pm 0{\cdot}0004$ grm.) was not greater than the growth in the non-aerated ones ($0{\cdot}0036 \pm 0{\cdot}0005$ grm.). This indicates that aeration favours growth, not by oxidizing waste products, but by keeping down the production of waste products.

Zone Formation by *Verticillium albo-atrum*.

In connexion with the experiments in temperature, it was found that the fungus formed zones regularly at a certain temperature, viz. 25° C. in all media. Fig. 11 shows zonation in Coon's medium with agar, and Fig. 12 zonation in corn-meal agar.

An attempt has been made to discover some of the conditions affecting ring formation. It has been studied in three different relations, viz. the medium, heat, and light.

It has been mentioned before that this fungus always causes the medium to become alkaline. Coon's normal, normal with 0·3 per cent. HCl, and normal with 0·3 per cent. NaOH, as well as Coon's N/2, N/4, and a fourteen days stale liquid, were plated with agar. These were inoculated and incubated at 14°, 16°, 18°, 21°, 22·5°, 25°, and 26° C., but zones appeared at 25° C. only, and the zoning in half-normal, quarter-normal, stale medium, and alkaline medium was more marked than in the others. Though the amount of growth at 18° C. was the same as that of 25° C., no rings appeared at the lower temperature. A study of a graph showing the daily spread in an agar colony at the two temperatures exhibits the lines crossing and recrossing many times, showing that the growths at these temperatures are the same. The total growths are also the same. If equal amounts of growth produce equal amounts of waste products, and staling products are the cause of zone formation, then we should expect rings equally at 18° C., as at 25° C. But no rings appeared at 18° C.

The rings at 25° C. do not appear successively as growth proceeds, as in *Penicillium*. The colony grows uniformly for a certain time, and then suddenly zones appear in the mycelium already formed, which had previously

shown uniform growth. Zones generally appear by the end of the second week, when an area of 45–50 mm. in diameter has appeared. Zones appeared at a lower temperature than 25° C. only when the Petri dishes were continuously lighted. Thus ordinary Petri dishes were kept at 21°–23° C. under continuous illumination, with some plates covered with black paint to prevent the entry of light. Zoning appeared only in the illuminated dishes,

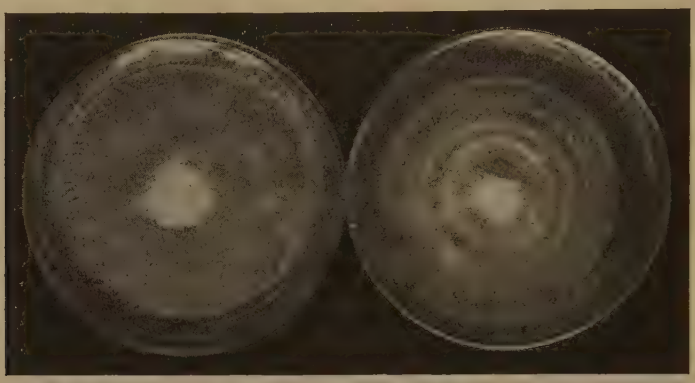

FIG. 11. Two cultures of *V. albo-atrum* on Coon's medium plus agar. That on the left was incubated at 22·5° C. and shows no zonation, that on the right at 25° C. and shows well-marked zonation.

FIG. 12. A culture of *V. albo-atrum* on corn-meal agar at 25° C.; zonation is clearly visible.

which definitely shows that it was the effect of light that brought about zoning at this lower temperature. Zones failed to appear in the dark even at 25° C. when the media was acidified to a pH of about 2·8, but the same medium produced zones when exposed to light. Normal Coon (pH 4·3) produces zones at 25° C., but these are not very prominent. Very marked zone formation takes place at 25° C. when a fourteen days stale medium (pH 5–5·2) is used. It is the same for 21°–23° C. in the light. At 25° C. in

darkness and 21°–23° C. lighted, ring formation is very marked when about 3 per cent. NaOH is added (pH 7·0). In connexion with zone formation at a lower temperature in light, it is noteworthy that the rings do not appear suddenly as at 25° C. in darkness, but gradually one after another from the very beginning. The results now may be tabulated as follows:

Medium.	22·5° C. *Darkness.*	21°–23° C. *Light.*	21°–23° C. *Darkness.*	25° C. *Darkness.*
Coon's normal	–	+	–	+
Coon's ½ normal	–	+	–	+
Coon's ¼ normal	–	+	–	+
Coon's 14 days stale	–	+	–	+
Coon's normal with 0·3 % HCl	–	+	–	–
Coon's normal with 0·3 % NaOH	–	+	–	+

At 20° C. and below this temperature, light produced no zonation in any medium.

We may now conclude that an alkaline medium favours ring formation (up to pH 7·0, or just over it), but if the medium becomes very alkaline (pH 8·0), as in a very stale six months old medium, the fungus grows, but no rings are formed. Also that *in the dark* zone formation is practically confined to 25° C., which, however, can be lowered slightly by exposure of the culture to light.

Zone formation has been held to be due to a periodic external factor, e.g. alternation of light and darkness; but in these experiments rings were formed not only in constant darkness but also under continuous illumination.

It has been claimed that the production of staling substances is the cause of zone formation. *Verticillium*, however, grows best at 22·5° C., so should then form most waste products, yet no zones are formed in darkness at that temperature, although they appear under similar conditions at 25° C. The way in which the zones appear suddenly at 25° C. does not support the alkali diffusion theory of Munk (3). Also Moreau's (4) view of alternation of light and darkness cannot be applicable as already stated. Hedgecock (5), in his experiments on zonation with *Cephalothecium*, found light to be the determining factor, and showed that variation of temperature did not cause zonation; he showed also that no zonation occurred in darkness, and that light of different wave length had different actions on zone formation. Similar experiments were carried out by Knischewsky (6) with *Penicillium luteum*. Munk (7) had also found that on exposure to light zonation develops, but not at constant temperature in darkness. He had found that increase in the amount of nutrient makes the zones narrower and tends to diminish coremium formation.

Plates were grown at 25° C. with the medium 6 mm. and 12 mm. thick,

Coon's normal medium agar being employed. In twenty days' time the total growths were as follows:

In medium 6 mm. thick . . Diameter of mycelial area 80 mm.
In „ 12 „ „ . . „ „ „ „ 60 mm.

The rings were more closely packed in the thicker medium (Fig. 4).

Summary.

Verticillium albo-atrum, B. et R., grows on a large variety of media, and always renders the medium alkaline.

The asparagin of Coon's medium can be markedly reduced without much affecting the growth of the fungus; on the other hand, reduction in the concentration of the maltose in the medium markedly reduces the growth.

The optimum temperature for growth is 22·5° C., the maximum 30° C., and the minimum 10° C.

Aeration in liquid media markedly increases not only the rate of growth but also the total amount of growth in a given volume of medium.

Aeration appears to increase growth by reducing the production of waste products, rather than by removing (oxidizing?) waste products already formed.

The rate of surface 'spread' of a culture on a solid medium was compared with the rate of increase of material as measured by dry-weight production. The rate of 'spread' as a measure of actual production of fungal material was found to be extremely untrustworthy. It gives, however, a satisfactory measure of the effect of different temperatures on the rate of growth in a medium of *constant composition and constant thickness.* When, however, different media are concerned the same rate of surface spread may be associated with extremely different rates of mycelium production.

In surface 'spread' the fungus follows approximately the compound interest law, but the dry-weight production there is more nearly a linear relation of time.

For cultures on solid media in the dark, zonation is confined to a temperature of about 25° C.; there is no zonation at 24° C. or 26° C. In the light, however, such cultures will show zonation at about 23° C.

The zones are more closely packed in a culture on a thick layer of medium than in that on a thin layer.

There is no evidence that accumulation of waste products in any way favours zone formation.

In conclusion the writer wishes to thank for his valuable help Professor V. H. Blackman, at whose suggestion this work was undertaken.

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